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Helicobacter pylori infection in relation to *E-cadherin* gene promoter polymorphism and hypermethylation in sporadic gastric carcinomas

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Abstract

AIM: To study *Helicobacter pylori* (*H pylori*) infection in relation to *E-cadherin* (*E-cad*) promoter polymorphism and hypermethylation in GCs.

METHODS: Specimens were taken from representative cancerous lesions and adjacent non-cancerous epithelia of 67 resected GCs. *H pylori* was detected by real-time PCR of the *cagA* gene from non-neoplastic epithelium. *E-cad* promoter polymorphism and hypermethylation were determined by restriction fragment length polymorphism analysis and methylation-specific PCR, respectively. Expression of *E-cad* protein was determined by immunohistochemistry.

RESULTS: *H pylori* was found in 57% of patients with GC. *H pylori* infection was more frequently found in tumors with the -160C/C genotype than those with the -160C/A and -160A/A genotypes (74% *vs* 47%, $P = 0.02$). *H pylori* infection was associated with *E-cad* methylation in non-neoplastic epithelium; however, no significant difference in *H pylori* was observed between methylated and unmethylated cancerous lesions.

CONCLUSION: Patients with the -160C/C genotype might require *H pylori* infection to promote the inactivation of *CDH1*, suggesting that *H pylori* infection might affect GC in an initial stage because polymorphism is germ line. Mechanism of hypermethylation of *CDH1* promoter in GC is complex, and *H pylori* infection might affect it in an initial stage.

Key words: *H pylori*; *E-cadherin*; -160 C→A polymorphism; Hypermethylation; Gastric carcinoma

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INTRODUCTION

E-cadherin (E-cad, also known as CDH1) is a member of a family of transmembrane glycoproteins expressed on epithelial cells and is responsible for calcium-dependent cell-to-cell adhesion[1]. E-cad forms complexes and connects actin filaments with α-, β-, and γ-catenins[2,3], which is crucially involved in neoplastic transformation and metastasis[4,5]. Loss of cell adhesion may contribute to loss of contact inhibition of growth, which is an early step in the neoplastic process. Furthermore, loss of cadherin activity may result in cancer cell detachment and metastasis[6,7].

Gastric carcinogenesis is a multistep process with morphological progression involving multiple genetic and epigenetic events, whereas *CDH1* is an important putative tumor suppressor gene. In gastric carcinoma (GC), the percentage reduction in E-cad expression varies from 17% to 92%, and is more frequent in diffuse type carcinomas than in intestinal types[8-13]. Germ-line mutation of the *CDH1* gene is found in familial GCs[14,15]. Somatic mutations of *E-cad* are found in more than 50% of gastric carcinomas of the diffuse type[16-18]. In addition to the classic two-hit inactivation mechanism, *CDH1* can be silenced by cytosine-guanosine (CpG) sequence methylation in GC[17,18]. Moreover, Li *et al*., reported that -160C/A polymorphism has a direct effect on the transcriptional regulation of CDH1[19].

Helicobacter pylori (*H pylori*) is the single most important

etiological factor for gastric cancer development. The strongest evidence comes from three independent, nested case-control studies in which pre-existing infection markedly increased the risk of GC[20-22]. It is estimated that approximately 60% of all GC cases can be attributed to *H pylori* infection[20]. However, there are few data in the molecular profiles of E-cad of *H pylori*-positive and *H pylori*-negative GCs[23].

El-Omar *et al*.[24], reported that interleukin-1 polymorphisms that cause the up-regulation of interleukin-1β with *H pylori* infection are associated with an increased risk of GC. Moreover, Hmadcha *et al*.[25], found that interleukin-1β might induce gene methylation through the production of nitric oxide and the subsequent activation of DNA methyltransferase. It is possible that *H pylori* infection induces methylation through the production of interleukin-1β. In this study, we investigated the relationship between *H pylori* infection and *CDH1* methylation. Since -160C/A polymorphism is germ line, we also investigated the relationship between *H pylori* infection and polymorphism, to find if *H pylori* infection affects tumorigenesis in the initial stage.

MATERIALS AND METHODS

Patients and samples

The specimens were surgically obtained from 67 patients with GC between 2000 and 2002, at the Division of General Surgery, Department of Surgery, Tri-Service General Hospital, Taipei, Taiwan. None of the subjects received preoperative anti-cancer therapy. Clinical information was obtained from medical records. Samples were taken from representative cancerous lesions and the corresponding non-cancerous epithelia. All tumor DNA samples were obtained by microdissection from 5-μm-thick hematoxylin and eosin-stained paraffin-embedded tissue sections[26]. Non-cancerous DNA was extracted from tissues which were flash-frozen in liquid nitrogen and stored in -80 ℃. All 67 samples were classified according to the Lauren criteria[27]: 26 as intestinal, 41 as diffuse types. The tumors were staged at the time of surgery using the standard criteria for TNM staging, with the unified international gastric cancer staging classification[28].

H pylori detection with real-time PCR

Real time PCR amplification of the *cagA* gene was conducted by adding 62.5 ng of DNA from stomach normal tissues isolates to 20 μL reactions containing 10 μL of 2X QuantiTect SYBR Green PCR Master Mix (QIAGEN, Hilden, Germany), 0.5 μmol/L S1227 Sense Primer (nt 154-175: 5'-GATAACAGGCAAGCTTTTGAGG-3'), 0.5 μmol/L AS1576 Antisense Primer (nt 503-583:5'-CTGCAAA-AGATTGTTTGGCAG-3') and 0.4 U of Uracil-N-glycosylase (New England Biolabs, Boston, USA).

Real-time PCR amplification was carried out in a LightCycler™ (Roche, Basel, Switzerland). After a preincubation step at 50 ℃ for 2 min, 95 ℃ for 15 min in order to activate the Uracil-N-glycosylase and HotStar Taq DNA polymerase, amplification was performed during 65 cycles including denaturation (94 ℃, 15 s with a temperature transition rate of 20 ℃/s), annealing (60 ℃, 25 s, with a temperature transition rate of 20 ℃/s) and extension (72 ℃, 18 s with a temperature transition rate of 20 ℃/s). A single fluorescent signal was obtained once per cycle at the end of the extension step with detection channel F1. After amplification, melting curve analysis was performed on the products by heating to 95 ℃ for 0 s, cooling to 65 ℃ for 15 s, followed by a temperature increase to 95 ℃ with a temperature transition rate of 0.2 ℃/s while continuously collecting the fluorescent signal.

To analyze the data of the real-time PCR assay, the derivative melting curves were obtained with the LightCycler data analysis software, version 3.5 (Roche). The melting temperature (*T*m) of the melting curve in each sample was used to detect the *H pyloric cagA* gene.

Restriction-fragment length polymorphism analysis to identify nucleotide changes at -160 of the CDH1 promoter

The -160 polymorphic site contained either a C or a A residue. The tumor type was determined by *Bst*EII digestion of the PCR products amplified using the primer set 5'-TGATCCCAGGTCTTAGTGAG-3' (upstream) and 5'-AGTCTGAACTGACTT CCGCA-3' (downstream). The 318-bp PCR product was cut into two fragments (208 and 110 bp) it contained the A residue. To ensure that the observed polymorphism was specific and not an experimental artifact, the results were confirmed by direct DNA sequencing.

Methylation-specific PCR and bisulfite-modified genomic sequencing to detect promoter hypermethylation of CDH1

The bisulfite modification procedure was carried out by the Zymo's EZ DNA Methylation Kit (Zymo Research, USA). One microliter of bisulfite-modified DNA was amplified in a total volume of 25 μL containing 1×PCR Buffer II, 2 mmol/L $MgCl_2$, 0.25 mmol/L deoxynucleotide triphosphate (dNTPs), 0.2 μmol/L each primer, and 0.04 U of Ampli Taq Gold™ DNA polymerase (Applied Biosystems, USA) at 95 ℃ for 30 s, the specific annealing temperature for 30 s, and 72 ℃ for 45 s. Water was used as a negative control. Seven millimole per liter/L of PCR product was directly loaded onto 3% agarose gel stained with ethidium bromide and directly visualized under UV illumination. Samples were scored as methylation, when there was a clearly visible band on the gel with the methylation primers[29].

Immunohistochemical staining and evaluation of E-cad expression

In immunohistochemical studies, sections (5 μm thick) from fixed, paraffin-embedded tumors were reacted with monoclonal anti-E-cad antibody (Cappel, Aurora, OH, USA), then with secondary antibody, and the signal was detected using an avidin-biotin complex and diaminobenzidine (DAB) kit (Vector Laboratories, Burlingame, CA, USA). DAB produced a yellowish brown stain, if the sample was positive. If more than 90% of the tumor cells exhibited intense membranous staining similar to that of normal cells, the result was considered positive (++). If the staining intensity was demonstrably reduced in comparison with that of normal cells and/or the staining pattern was heterogeneous (10-90% positive), the result was deemed to be weakly

positive (+). If E-cad expression was completely lost or positive in less than 10% of cells, the result was defined as negative (-).

Statistical analysis

Analyses were performed using S-Plus®2000 for Windows Statistical Software (CANdiensten, Amsterdam, The Netherlands). The level of significance was set at $P<0.05$ for all tests. Continuous variables are expressed as mean±SD, and were tested using Student's *t* test; categorical variables were tested using Fisher's exact test.

RESULTS

Of these 67 patients, 45 were men, and 22 were women. Their median age was 68.56 years (range, 31-88 years). All patients underwent surgical resection of the primary tumor. Twenty-six and 41 tumors were of the intestinal and diffuse histotypes, respectively. Reduced E-cad expression was observed in 88% (36/41) of diffuse type tumors and in 50% (13/26) of intestinal type tumors ($P = 0.0015$). Representative examples of immunohistochemical staining for E-cad expression in non-cancerous epithelium (++) and diffuse type tumor (-) are shown in Figures 1A and B.

H pylori infection was detected by real-time PCR (iCycler iQ Real-Time Quantitative System) for *H pylori cagA* gene in non-cancerous gastric epithelial samples adjacent to tumors. *H pylori* was found in 57% of GC patients. No significant difference in *H pylori* infection was observed between intestinal and diffuse type tumors (23/41, 56% *vs* 15/26 58%). There was no significant difference in reduced E-cad expression between *H pylori*-positive tumors and *H pylori*-negative tumors (27/38, 71.1% *vs* 22/29, 76%).

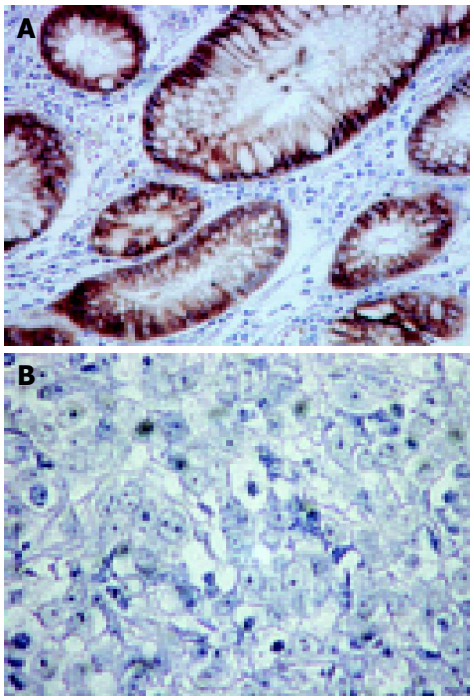

Figure 1 Immunohistochemical staining. **A**: Positive (++) immunohistochemical staining for E-cad expression in non-cancerous epithelium; **B**: Negative (-) immunohistochemical staining for E-cad expression in cancerous lesion in diffuse type tumor.

H pylori and -160C/A polymorphism

Among the 67 patients, 27 were genotype C/C (40%), 26 were genotype A/C (39%), and 14 were genotype A/A (21%) (Figure 2). There was no significant difference in the frequency of the C/A+A/A genotypes between tissues with normal and reduced E-cad expression (13/18, 72% *vs* 27/49, 55%). Interestingly, analyzing 67 available cases of *H pylori* infection and polymorphism status, *H pylori* infection was more frequently observed in tumors with C/C genotype of *CDH1* promoter than in C/A+A/A genotype tumors (20/27, 74% *vs* 18/40, 45%; $P = 0.02$, Table 1).

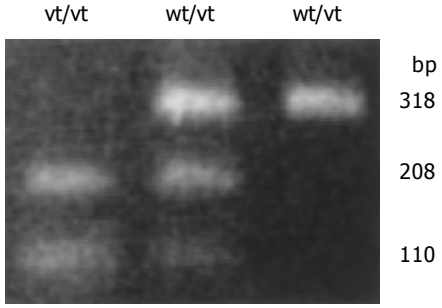

Figure 2 RFLP analysis of genetic polymorphism of the 160 site of the *E-cad* promoter. The C/A polymorphism was differentiated by *Bst*EII digestion of PCR products homozygous for the wild-type (high-activity) allele (wt/wt, CC genotype), heterozygous for the variant (low-activity) allele (wt/vt, CA genotype), and homozygous for the low-activity allele (vt/vt, AA genotype).

H pylori infection and hypermethylation in non-cancerous gastric epithelia

Promoter hypermethylation was found in 87% non-cancerous gastric epithelia of 67 GC patients (Figure 3A). Hypermethylation was frequently associated with *H pylori* positive than *H pylori* negative (36/38, 95% *vs* 22/29, 76%, $P = 0.025$, Table 1).

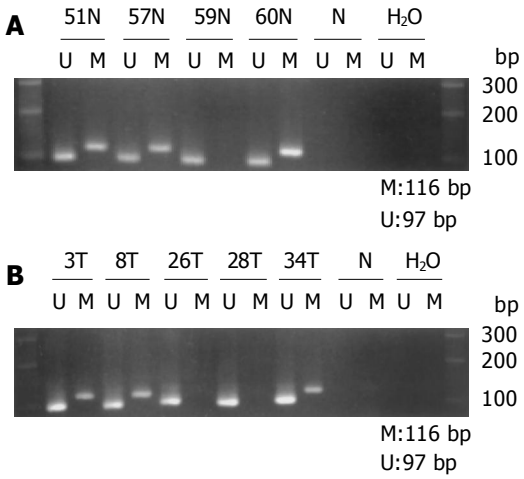

Figure 3 Gel electrophoresis picture demonstrating aberrant methylation in E-cad. Primer sets used for simple amplification are designed as unmethylated (U), methylated (M), unmodified DNA (N). Water is used as a negative control (H_2O). Molecular weight marker is 100-bp DNA ladder. **A**: Methylation in non-cancerous epithelium. Samples 51, 57, and 60 N are methylated. Sample 59 N is unmethylated; **B**: Methylation in cancerous lesion. Samples 3, 8, and 34 T are methylated. Samples 26 and 28 T are unmethylated.

H pylori infection and hypermethylation in cancerous lesions

Results from MSP indicated that the *CDH1* promoter was hypermethylated in 45 (67%) of 67 GCs (Figure 3B). Hypermethylation was more frequent in diffuse type tumors (76%, 31/41) than in intestinal type tumors (50%, 13/26; $P = 0.03$). Furthermore, hypermethylation was more frequent in GCs with reduced E-cad expression than normal expression (37/45, 82% *vs* 12/22, 55%, $P = 0.02$). No significant difference in *H pylori* infection was observed between methylated and unmethylated tumors (26/45, 58% *vs* 12/22, 55%, Table 1).

Table 1 *H pylori* infection in association with histotypes and E-cad expression profiles in GC

	H pylori (+) (%)	*H pylori* (–) (%)	*P*
Lauren's			
Intestinal type	23 (56.1)	18 (43.9)	
Diffuse type	15 (57.7)	11 (42.3)	NS
Promoter polymorphism			
C/C	20 (74.1)	7 (25.9)	
C/A+A/A	18 (45)	22 (55)	0.02
Methylation (noncancerous)			
Methylated	36 (94.7)	22 (75.9)	
Unmethylated	2 (5.3)	7 (24.1)	0.025
Methylation (cancerous)			
Methylated	26 (68.4)	19 (65.5)	
Unmethylated	12 (31.6)	10 (34.5)	NS
IHC			
Normal (++)	11 (28.9)	7 (24.1)	
Reduced (+, –)	27 (71.1)	22 (75.9)	NS

NS: not significant.

DISCUSSION

H pylori is responsible for the pathogenesis of atrophic gastritis and intestinal metaplasia[30,31]. Epidemiological studies have indicated that infection with *H pylori* is a risk factor for GC[20-22]. Moreover, Mongolian gerbils develop chronic gastritis, intestinal metaplasia, and adenocarcinoma after inoculation with *H pylori* into the stomach. However, the link between *H pylori* infection and the molecular mechanism of human gastric carcinoma remains to be investigated. Lim *et al*., reported that the increased expression of cell adhesion molecules (galectin 1, aldolase A, integrin α5, LMO7) and the decrease in E-cad expression induced by *H pylori* might contribute to cell adhesion, invasion, and possibly cell proliferation in gastric epithelial cells[32]. Kitadai *et al*., reported that coculture with *H pylori* increased the expression of interleukin-8, vascular endothelial growth factor (VEGF), angiogenin, uPA, and MMP-9 and increased angiogenic and collagenase activities in gastric carcinoma cells[33]. Sharma *et al*., reported that the activation of interleukin-8 gene expression by *H pylori* is regulated by the transcription factor, nuclear factor-kappa B, in gastric epithelial cells[34]. Akhtar *et al*., reported that promoter methylation regulates *H pylori*-stimulated cyclooxygenase-2 expression in gastric epithelial cells[35]. However, *in vivo* data on the differences in the molecular profile (ras, *MDM2*, c-erbB-2, cyclin D1, p53, *CDH1*) of *H pylori*-positive and *H pylori*-negative gastric carcinomas are almost non-existent[23].

In this study, *H pylori* was found in 57% of patients with GC. No significant difference in *H pylori* infection was observed between intestinal and diffuse type tumors (56% *vs* 58%). This is consistent with the current view that 60% of all GC cases are related to *H pylori* infection, irrespective of the histological tumor type[20,23].

H pylori and promoter polymorphism

Li *et al*., reported that the A allele of the -160C/A promoter polymorphism altered transcriptional binding, resulting in a reduction in transcriptional efficiency of 68% relative to that of the C allele[19]. In this study, *H pylori* was significantly more frequent in the C/C genotype of *CDH1* promoter than in the C/A+A/A genotypes. The C allele shows better transcriptional activity than the A allele, so patients with the C/C type may require *H pylori* infection to promote the inactivation of *CDH1*. Since -160C/A polymorphism is germ line, *H pylori* infection might affect *CDH1* inactivation in an early, possibly preneoplastic stage.

H pylori and promoter hypermethylation

In this study, hypermethylation of the *CDH1* promoter was present in 67% GCs, and was more frequent in diffuse type tumors (76%) than in intestinal type tumors (50%, $P = 0.03$). *CDH1* promoter hypermethylation was associated with decreased immunohistochemical expression of E-cad (82% *vs* 55%, $P = 0.02$). These data are similar to the results reported by Tamura *et al*., and Graziano *et al*.[37], which indicated that *CDH1* promoter methylation may play a major role, together with mutations or deletions, in causing inactivation of the *CDH1* gene in GC, especially in diffuse type tumors.

Chan *et al*.[18], investigated *CDH1* hypermethylation in the gastric mucosa of 35 patients with dyspepsia and found that hypermethylation was age-related and associated with *H pylori* infection. In our study, hypermethylation was found in 87% non-cancerous gastric epithelia of 67 GC patients and frequently associated with *H pylori* positive than *H pylori* negative epithelia. Therefore, our results of promoter hypermethylation of *E-cad* in non-cancerous gastric epithelia are compatible with hypothesis proposed by El-Omar *et al*.[24], and Hmadcha *et al*.[25]. However, we observed no association between *H pylori* infection and *CDH1* methylation in the gastric cancerous epithelia. It seems that *H pylori* is unrelated to *CDH1* methylation in GC. However, on the basis of current knowledge, *H pylori* should be considered as a factor facilitating the multifactorially determined process of gastric carcinogenesis, but by itself does not initiate or trigger the carcinogenic process (the "hypothesis of no return")[23,38]. Moreover, few *H pylori* are detected in cancerous epithelia in the human stomach. Under *in vivo* conditions, *H pylori* infects the non-cancerous epithelia surrounding the tumor and up-regulates methylation, and may indirectly stimulate hypermethylation in GCs at the tumor-normal tissue interface[33]. In addition, DNA methylation is catalyzed by DNA methyltransferase, and DNA CpG islands can be demethylated by MBD2[39]. *H pylori* infection might induce activation of DNA methyltransferase. However, Oue *et al*., reported that the mechanism of

CpG island hypermethylation is complex in the GC microenvironment and may not result from a simple transcriptional up-regulation of methyltransferase or down-regulation of *MBD2*[40]. Thus from the results of this study and the above statement, we conclude that the mechanism of hypermethylation of *CDH1* in GC is complex, and *H pylori* might affect hypermethylation in an initial stage.

In conclusion, patients with the -160C/C genotype might require *H pylori* infection to promote the inactivation of *CDH1*. *H pylori* infection might affect *E-cad* promoter hypermethylation of GC in an initial stage. Therefore, *H pylori* infection should be considered as a factor that facilitates a multifactorially determined process of gastric carcinogenesis in an initial stage.

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